



**BIODEGRADATION OF ORGANOPHOSPHATE CHEMICAL
WARFARE AGENTS BY ACTIVATED SLUDGE**

Steven J. Schuldt, Capt, USAF

AFIT/GES/ENV/12-M04

**DEPARTMENT OF THE AIR FORCE
AIR UNIVERSITY**

AIR FORCE INSTITUTE OF TECHNOLOGY

Wright-Patterson Air Force Base, Ohio

APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

The views expressed in this thesis are those of the author and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the United States Government. This material is declared a work of the United States Government and is not subject to copyright protection in the United States.

BIODEGRADATION OF ORGANOPHOSPHATE CHEMICAL WARFARE
AGENTS BY ACTIVATED SLUDGE

THESIS

Presented to the Faculty

Department of Systems and Engineering Management

Graduate School of Engineering and Management

Air Force Institute of Technology

Air University

Air Education and Training Command

In Partial Fulfillment of the Requirements for the
Degree of Master of Science in Environmental Engineering and Science

Steven J. Schuldt, B.S.

Captain, USAF

March 2012

APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

BIODEGRADATION OF ORGANOPHOSPHATE CHEMICAL WARFARE
AGENTS BY ACTIVATED SLUDGE

Steven J. Schuldt, B.S.
Captain, USAF

Approved:

/signed/
LeeAnn Racz, Maj, USAF, Ph.D. (Chairman)

8 Mar 2012
Date

/signed/
Dirk P. Yamamoto, Lt Col, USAF, Ph.D. (Member)

8 Mar 2012
Date

/signed/
Edward Hess, M.S., M.S.ChE, USAF (Member)

8 Mar 2012
Date

Abstract

Organophosphates (OPs) have been widely used as Chemical Warfare Agents (CWAs) as well as pesticides since World War II and still remain a threat to national security. While efforts have been taken at military installations and civilian communities to secure these chemicals and prevent their misuse, a determined adversary could still obtain and deploy them to injure, kill or instill terror. The lethal properties of this group of compounds are primarily owed to their irreversible inhibition of the enzyme acetylcholinesterase (AChE) and thus may alter the human nervous system or affect the hormonal balance of children in particular.

In the event of a chemical incident, standard operating procedures dictate that contaminated personnel be decontaminated. Often times, decontamination is accomplished with water. Many communities plan for this decontamination water to be sent to the local municipal wastewater treatment plant. However, the fate of these compounds in a municipal wastewater treatment plant is largely unknown. If the compounds cannot be degraded, they will enter surface water bodies with plant effluent or waste sludge.

This research examined the fate of ethyl methylphosphonic acid (EMPA), a hydrolysis product of VX, in bench-scale sequencing batch bioreactors that simulated a municipal activated sludge wastewater treatment system. Results show that CWA may pass through an activated sludge wastewater treatment system largely unchanged as EMPA did not sorb to the biomass and only 28% of the initial 1 mg L⁻¹ concentration was degraded.

For my family, Michelle, Austin and Emmalyn

Acknowledgments

This work would not have been possible without the helpful hand of many people. In the sake of brevity, I have chosen to highlight those whose contributions were the greatest. First, I would like to thank Major LeeAnn Racz for her exceptional guidance and mentoring. You went above and beyond what is required of an advisor and your friendship in something I will never forget. To Lieutenant Colonel Yamamoto and Ed Hess, thank you both for being a part of my committee. Your insights and perspective helped tremendously. To Stuart Willison, thank you for developing a method to analyze my samples and for completing the analysis at your laboratory. The data you provided helped make this all possible. To my wife, Michelle, thank you for your constant love and support. Thank you for always being available as a listening ear no matter how boring my presentations were or how little you understood. I especially thank you for bringing our beautiful daughter into this world and doing so much to take care of her during the last six months. I thank God for the woman and mother you are. To my great friend, Captain Justin DeLorit, thank you for all of your hard work and for making this entire process much more enjoyable. I considerate myself so fortunate to have met you and am blessed by the opportunity to work with you again. Last, but most importantly, I thank my Lord and Savior, Jesus Christ. God has faithfully provided me with more strength and encouragement than I could possibly imagine during my time here. By his grace, I leave a better man, a better father and a better friend.

Steven J. Schuldt

Table of Contents

	Page
Abstract.....	iv
Acknowledgments.....	vi
Table of Contents.....	vii
List of Figures.....	ix
List of Tables	x
 I. Introduction	 1
Background	1
Problem Statement	13
Research Questions	13
Scope and Approach	14
Significance.....	14
Preview.....	15
 II. Scholarly Article	 16
Abstract	16
Introduction.....	16
Materials and Methods.....	18
<i>Sequencing Batch Reactor Operation</i>	18
<i>Solid and Liquid Phase</i>	20
<i>UPLC/MS-MS</i>	21
<i>Sorption Kinetics</i>	21
<i>Sorption Isotherm</i>	22
<i>Biodegradation</i>	22
<i>Biodegradation with Inhibition of Nitrification</i>	23
<i>Other Analytical Methods</i>	24
Results and Discussion.....	24
<i>Sorption Kinetics and Equilibrium Isotherms</i>	24
<i>Biodegradation</i>	25
Conclusions	27
 III. Conclusions.....	 28
Chapter Overview	28
Review of Findings	28
Significance of Research.....	28

	Page
Limitations	29
Future Research.....	30
Summary	31
Appendix A. UPLC/MS-MS Calibration Curves	32
Bibliography	34

List of Figures

	Page
Figure 1: The mechanism of action of AChE (adapted from Katzung, Masters, & Trevor, 2011)	2
Figure 2: Primary Hydrolysis Pathways of VX in the Environment (Munro, 1999).....	10
Figure 3: Wastewater Treatment Process (Ohio State University Extension, 2012).....	11
Figure 4: SBR setup	20
Figure 5: EMPA Biodegradation: 1 mg L ⁻¹ activated sludge at 25° C with nitrifiers active	25
Figure 6: EMPA Biodegradation: 1.2 mg L ⁻¹ activated sludge at 25° C with nitrifiers inhibited	26
Figure 7: Calibration curve for sorption kinetics	32
Figure 8: Calibration curve for sorption equilibrium isotherm.....	32
Figure 9: Calibration curve for degradation with nitrifiers active	33
Figure 10: Calibration curve for degradation with nitrifiers inhibited	33

List of Tables

	Page
Table 1: Signs and Symptoms of Acute Poisoning with Anticholinesterase Compounds (Casarett, Doull, & Klaassen, 2007).....	3
Table 2: Identity and Chemical and Physical Properties of Chemical Warfare Agents (Munro, 1999).....	8

BIODEGRADATION OF ORGANOPHOSPHATE CHEMICAL WARFARE AGENTS BY ACTIVATED SLUDGE

I. Introduction

Background

Organophosphates are esters of phosphoric acid (Szinicz, 2005). First developed in France in the mid-19th century, organophosphates have a number of important uses including insecticides, flame retardants, softeners, plasticizers, lubricating oil additives and emulsifiers, but they are most known for the danger they present as highly toxic nerve agents (Szinicz, 2005).

The first highly toxic organophosphate, tetraethyl pyrophosphate (TEPP), was synthesized in the De Clermont laboratory in France in the mid-19th century, but the high toxicity of this class of compounds was not recognized until the 1930s in Germany (Szinicz, 2005). Interest in the synthesis of organophosphates was originally focused on the development of insecticides, but the German Ministry of War saw the potential of organophosphates for military purposes after receiving samples of tabun and sarin in 1937 (Szinicz, 2005). All patent applications concerning these agents, approximately 200 in total, were declared secret. Of these, only tabun, sarin and soman were considered relevant chemical warfare agents (Holmstedt, 1963; Robinson & Leitenberg, 1971).

Research was also conducted in English and American laboratories during World War II, but it was only after the war, when the extent of German research became known, that nerve agents were intensively researched and viewed as having military significance (Szinicz, 2005). The United States, England, France and the Soviet Union all took a great interest in the development and production of nerve agents (Szinicz, 2005). In

1961, research ultimately led to the development of VX, the most effective chemical warfare agent ever produced, which was a product of the combined research and investigational efforts made by British and US laboratories (Szynicz, 2005).

Nerve agent toxicity is caused by the inhibition of acetylcholinesterase (AChE), the enzyme responsible for the breakdown of the neurotransmitter acetylcholine (Talmage, 2007). Under normal conditions, acetylcholine bonds with a protein receptor and then quickly dissociates (Fox, 2009). It is then inactivated by acetylcholinesterase after it is released by the receptor protein (Fox, 2009). The hydrolysis products of this inactivation are acetate and choline (see Fig 1) (Fox, 2009). Inhibition of acetylcholinesterase by nerve agents results in the accumulation of acetylcholine at cholinergic synapses and the overstimulation of receptor proteins of the muscarinic and nicotinic type (Gallo & Lawryk, 1991; Lotti, 2000, 2001).

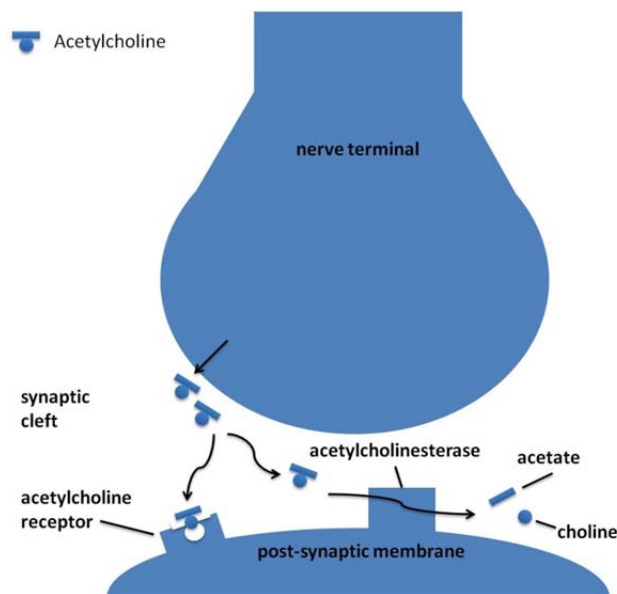


Figure 1: The mechanism of action of AChE (adapted from Katzung, Masters, & Trevor, 2011)

Depending on route and degree of exposure, symptoms of nerve agent exposure include increased sweating and salivation, profound bronchial secretion, miosis, diarrhea, tremors, fasciculation, and various central nervous system effects (Gallo & Lawryk, 1991; Lotti, 2000, 2001). When death occurs, it is most often due to respiratory failure due to inhibition of the respiratory centers in the brain stem, bronchoconstriction, increased bronchial secretion and flaccid paralysis of respiratory muscles (Gallo & Lawryk, 1991; Lotti, 2000, 2001). A complete list of signs and symptoms of acute nerve agent poisoning is available in Table 1.

Table 1: Signs and Symptoms of Acute Poisoning with Anticholinesterase Compounds (Casarett, Doull, & Klaassen, 2007)

Site and Receptor Affected	Manifestations
Exocrine glands (M)	Increased salivation, lacrimation, perspiration
Eyes (M)	Miosis, blurred vision
Gastrointestinal tract (M)	Abdominal cramps, vomiting, diarrhea
Respiratory tract (M)	Increased bronchial secretion, bronchoconstriction
Bladder (M)	Urinary frequency, incontinence
Cardiovascular system (M)	Bradycardia, hypotension
Cardiovascular system (N)	Tachycardia, transient hypertension
Skeletal muscles (N)	Muscle fasciculations, twitching, cramps, generalized weakness, flaccid paralysis
Central nervous system (M,N)	Dizziness, lethargy, fatigue, headache, mental confusion, depression of respiratory centers, convulsions, coma

M = muscarinic receptors; N = nicotinic receptors

Despite the vast amount of resources that were used to develop and produce chemical weapons from the 1930s to 1950s, their use has been limited in war. Additionally, the Chemical Weapons Convention (CWC), which came into effect on April 29, 1997, requires all member states to destroy their chemical weapon stockpiles and cease in the production, acquisition or transfer of chemical weapons (Richardson & Caruso, 2007). It is apparent that the risk of a nerve agent attack from a CWC-abiding nation is not likely a concern; however, the potential of nerve agent release or terrorist attack still exists as is evident by three major cases since 1984: the Iran-Iraq War, the Aum Shinrikyo terrorist attacks in Japan and the exposure of two US soldiers to sarin in Iraq in 2004.

The Iran-Iraq war began on September 22, 1980, when Iraq launched an invasion against Iran (Associated Press, 1990; Dunn, 1986; Rohrbaugh, Ward, & Yang, 1990; Spiers, 1989). The Iraqi army, having been trained and influenced by the Soviets, possessed organic chemical warfare units and many potential delivery systems (Associated Press, 1990; Dunn, 1986; Rohrbaugh et al., 1990; Spiers, 1989). The Iraqis first reported use of chemical weapons, used in a defensive effort to stop the human-wave-attack tactics of the Iranians, was in November 1980 (Associated Press, 1990; Dunn, 1986; Rohrbaugh et al., 1990; Spiers, 1989). The attacks were a success against an ill-prepared Iranian infantry and continued for many years. Iran notified the United Nations in 1983 that Iraq was using chemical weapons against its troops (Dunn, 1987; United Nations, 1986; UN panel, 1988). In response, the United Nations sent specialists to the area in 1984, 1986 and 1987 to verify the claims (Dunn, 1987; United Nations, 1986; UN panel, 1988). The United Nations concluded that Iraq was using chemical

weapons against the Iranians and their use appeared to be increasing (Dunn, 1987; United Nations, 1986; UN panel, 1988). It was also determined that mustard and tabun were the primary agents used and the primary delivery method was bombs dropped from airplanes (Dunn, 1987; United Nations, 1986; UN panel, 1988). Despite Iraq's use of chemical weapons, the war never reached a military conclusion. In total, approximately 5% of Iranian casualties were caused by chemical weapons (Hoffman, 1990).

The Aum Shinrikyo cult successfully planned and conducted the only case of a nongovernmental group manufacturing a nerve agent and using it against unprotected civilians (Hill, Kok, Mauroni, & Smart, 2008). Founded in 1987 by Shoko Asahara, Aum Shinrikyo, or the "Supreme Truth" held the belief that the world would end in a chemical warfare agent Armageddon (Hill et al., 2008b). The cult was well-financed and boasted a total membership of some 40,000 Japanese and Russians by 1995 (Hill et al., 2008b). Asahara began conducting small scale attacks in the early 1990s with anthrax which proved unsuccessful in causing casualties and instead turned his attention toward sarin in 1993 (Hill et al., 2008b). On June 27, 1994, the Aum conducted their first deadly sarin attack in the town of Matsumoto, about 200 miles northwest of Tokyo (Hill et al., 2008b). The target was three judges who were hearing a real estate lawsuit against the cult. When it became clear that the decision would likely go against the Aum, they decided to kill the judges (Hill et al., 2008b). The attack was conducted outside the judges' apartment complex using a modified refrigeration truck that held a heater, an electric fan and 30 kilograms of sarin. Seven people were killed and 144, including the three judges, were injured as a result of the attack (cns.miis.edu, 1996; Smithson & Levy, 2000)

The Japanese police planned to raid the Aum's facilities in March 1995 (Beaton et al., 2005). In an attempt to disrupt the raid, the Aum conducted their second terrorist attack, targeting Tokyo subway stations that served key governmental agencies (Beaton et al., 2005). Five teams of two cult members, each outfitted with bags containing 600 g of sarin, boarded three major subway lines (Beaton et al., 2005). The sarin was released when cult members punctured the bags with umbrellas (Beaton et al., 2005). In total, passengers at more than 15 subway stations were exposed, 12 people were killed, 54 were placed in critical condition and roughly 900 more were hospitalized (Beaton et al., 2005). In addition, some 5,500 "worried well" flooded the hospitals, completely overwhelming emergency response personnel (Beaton et al., 2005).

The most recent known exposure to nerve gas, and also the first reported exposure to American military personnel, occurred in Iraq in May 2004 when two US Army explosive ordnance soldiers came into contact with an old sarin shell, presumably from the Iran-Iraq war (McDonough, Newmark, & Sidell, 2008). The soldiers experienced mild sarin poisoning with the following symptoms: miosis, dim vision, increased nasal and oral secretions, mild dyspnea and acute memory disturbances (McDonough et al. 2008).

Many terrorism experts hold a common belief regarding the use of chemical weapons against noncombatants, "it's not a question of if, but when" (Hill, Hilmas, & Smart, 2008). There are four major reasons terrorists could naturally be drawn to the use of chemical warfare agents versus another method like biological or conventional weapons: their cost and stability, simplicity of production, pound for pound potency and fear factor (Hill et al., 2008a). Compared to biologicals, chemicals are readily available,

inexpensive and stable (Purver, 1995). For example, sarin could easily be produced by a moderately experienced chemist with access to the common chemicals chlorine and cyanide and the technology required via internet sources (Hill et al., 2008a).

Chemical agents, especially nerve agents, have a dramatic fear factor due to the symptoms they cause. Witnessing civilians violently convulsing on the ground can wreak havoc in an urban setting without the need of an explosion (Hill et al., 2008a). Additionally, chemicals are much more potent than conventional explosives on a pound for pound comparison (Hill et al., 2008a). All of these reasons lead experts to speculate that the use of chemical warfare agents could be very appealing to terrorists (Hill et al., 2008a).

Table 2 identifies many of the chemical and physical properties of the four most prevalent nerve agents, GA (tabun), GB (sarin), GD (soman) and VX. While each is highly toxic and potentially lethal as noted from the toxicological information above, VX stands apart as the most dangerous for three primary reasons. First, with an LD₅₀ [dosage (milligrams toxicant per 70 kilogram person) causing death in 50% of an exposed population] of 10 mg/70Kg, VX is the most lethal of the nerve agents. Second, with a hydrolysis rate (half-life) of 1,000 hours, VX is the most persistent nerve agent. Lastly, with a volatility of 10.5 mg/m³, VX is the least likely to enter a vapor form. This is a key fact for this research as it is investigating the degradation of nerve agents in wastewater. For these reasons, VX has been selected as the nerve agent of interest in this research.

Table 2: Identity and Chemical and Physical Properties of Chemical Warfare Agents (Munro, 1999)

Property/ Parameter	Agent			
	GA (Tabun)	GB (Sarin)	GD (Soman)	VX
Chemical formula	C ₆ H ₁₁ N ₂ O ₂ P	C ₄ H ₁₀ FO ₂ P	C ₇ H ₁₆ FO ₂ P	C ₁₁ H ₂₆ NO ₂ PS
Melting point	-50°C	-56°C	-42°C	-39°C
Boiling point	220-246°C	158°C	198°C	298°C
Density, liquid (g/mL)	1.073 at 25°C	1.102 at 20°C	1.022 at 25°C	1.008 at 20°C
Vapor pressure (mmHg 20 or 25°C)	0.037 at 20°C 0.07 at 25°C	2.10 at 20°C	0.40 at 25°C	0.0007
Volatility (mg/m ³)	610	22,000	3,900	10.5
Vapor density (air = 1)	5.6	4.9	6.3	9.2
Water solubility (g/L)	98 at 25°C	Miscible	21 at 20%	30
Hydrolysis rate (half-life)	8.5 hr (pH 7)	39 hr (pH 7)	45 hr (pH 6.6)	1,000 hr (pH 7)
Henry's constant (H, atm x m ³ /mol)	1.52 x 10 ⁻⁷	5.4 x 10 ⁻⁷	4.6 x 10 ⁻⁶	3.5 x 10 ⁻⁹
Log K _{ow}	0.384	0.299	1.824	2.09
Log K _{oc}	2.02	1.77	1.17	2.5
LD ₅₀ (mg/70Kg)	1,000	1,700	50	10

Hydrolysis of VX occurs via two pathways which are pH dependent and displayed graphically in Figure 2 (Munro, 1999). One hydrolysis pathway occurs at neutral pH, between 7 and 10 (Talmage, 2007). At these pH values, cleavage of the carbon-oxygen bond predominates which results in the formation of the environmentally stable S-(2-Diisopropylaminoethyl) methyl phosphonothioate (EA 2192) (Munro, 1999). EA 2192, like VX, inhibits acetylcholinesterase and is still very toxic (Munro, 1999). Its

intravenous toxicity is roughly equivalent to that of VX and its oral lethality is approximately an order of magnitude less (Munro, 1999). Fortunately, EA 2192 is not absorbed through the skin and it is highly unlikely to be inhaled, leaving the oral route of exposure as the only concern (Munro, 1999). Due to the persistence and toxicity of EA 2192, hydrolysis of VX between a pH of seven and ten is strongly discouraged.

The second hydrolysis pathway occurs in both acidic and alkaline conditions (Talmage, 2007). When VX is hydrolyzed at pH values less than six or greater than ten, cleavage of the phosphorus-sulfur bond predominates and results in the formation of ethyl methylphosphonic acid (EMPA) and diisopropylethyl mercaptoamine (DESH) (Munro, 1999). While no toxicity information is available on EMPA, it is structurally similar to isopropyl methylphosphonic acid (IMPA) and is likely to have the same low-to-moderate toxicity (Munro, 1999). Additionally, with a reference dose that is roughly 42,000 times greater than that of EA 2192 (Munro, 1999) (25 $\mu\text{g/kg/day}$ for EMPA versus 0.0006 $\mu\text{g/kg/day}$ for EA 2192), it is clear that degrading to EMPA is strongly preferred.

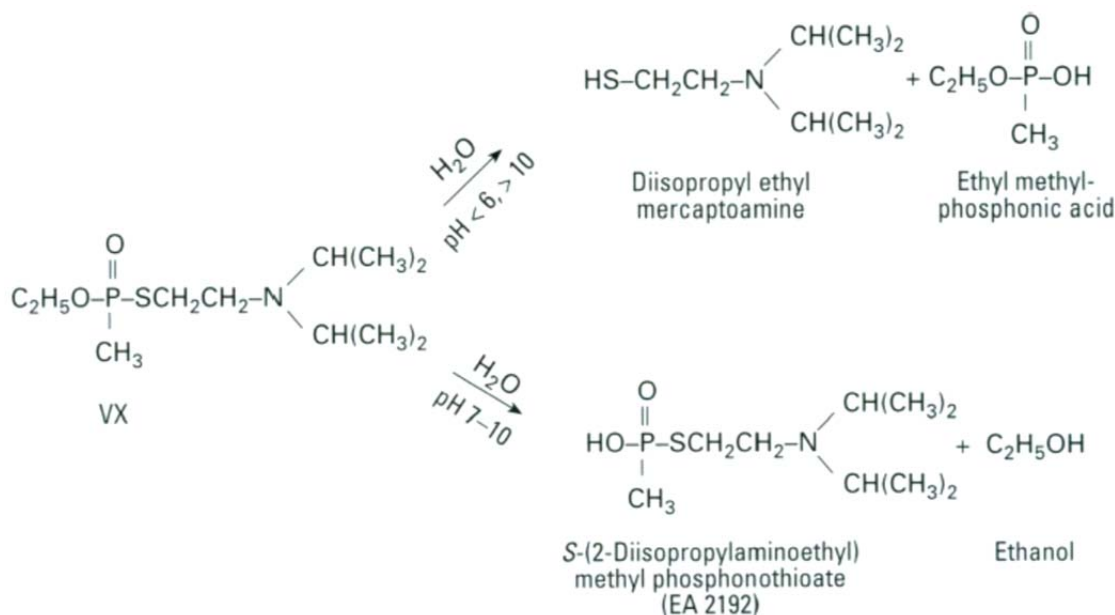


Figure 2: Primary Hydrolysis Pathways of VX in the Environment (Munro, 1999)

In the event of a chemical incident, standard operating procedures dictate that contaminated personnel, equipment and surfaces be decontaminated (Talmage, 2007). Water is often used for large scale decontamination of large open areas and personnel (Talmage, 2007). Because VX is one of the most difficult chemical warfare agents to destroy, it is often necessary to detoxify with copious amounts of aqueous bleach (Talmage, 2007). The addition of bleach typically raises the pH of the decontamination water to above ten which results in a hydrolysis pathway that leads to the formation of ethyl methylphosphonic acid, the surrogate being used in this research (Talmage, 2007).

Many communities plan for this decontamination rinse water to be sent to the local municipal wastewater treatment plant. However, the fate of VX and its hydrolysis products in a municipal wastewater treatment plant is largely unknown. Standard municipal wastewater treatment plants begin with screens and grit chambers which are

used for physical removal of larger debris and sediment (Fig 3). The water then typically enters an aerobic reactor containing activated sludge, which is a combination of flocculated biological growth and wastewater. The activated sludge is responsible for the biological treatment of the wastewater to include carbonaceous oxidation and, often times, nitrification (Droste, 1997).

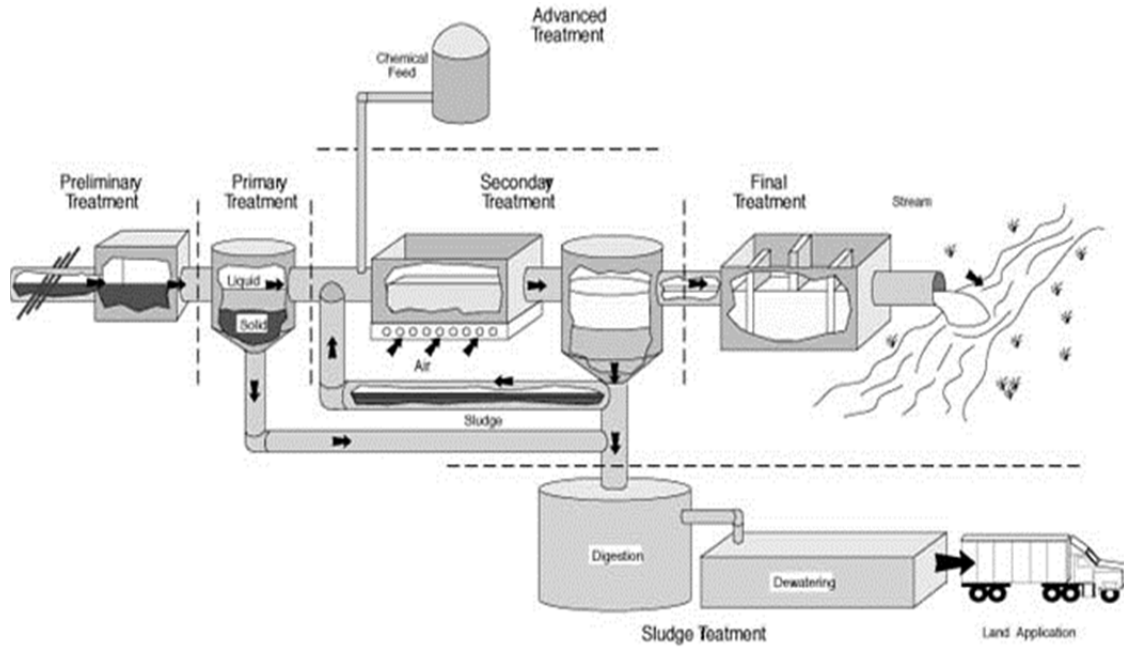
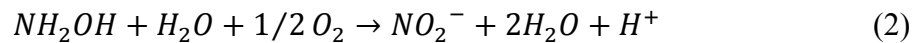
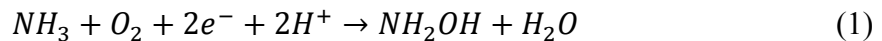


Figure 3: Wastewater Treatment Process (Ohio State University Extension, 2012)

Nitrification, the biological process by which ammonia is removed from wastewater, is often conducted at wastewater treatment plants and occurs via three steps summarized by equations 1-3.



Ammonia oxidizing bacteria (AOB) such as *Nitrosomonas* spp. and *Nitrosospira* are responsible for oxidizing ammonia (NH_3) to the intermediate, hydroxylamine (NH_2OH), via the ammonia monooxygenase (AMO) enzyme (Racz & Goel, 2009). AMO then catalyzes the hydroxylation of alkenes to produce primary and secondary alcohols by inserting oxygen into C-H bonds (Hyman & Wood, 1983; Hyman, Murton & Arp, 1988). This hydroxylation via AMO has previously been attributed to converting organic compounds such as estrogens into hydrophilic products essentially devoid of estrogenic activity as described by Vader et al. (2000). Thus, it was determined that estrogen degradation in nitrifying biomass could be contributed to cometabolism via AMO (Ren et al., 2007a; Shi et al., 2004; Vader et al., 2000). It is possible that AMO cometabolism could be involved with degradation of other organic compounds, such as OP CWA, as well.

Following biological treatment, water is then discharged into some water body. Additionally, the accumulation of flocculated biological material requires that a portion of the sludge periodically be wasted. For nitrifying activated sludge, the typical solids retention time (SRT) or sludge age is approximately 15-20 days (Metcalf & Eddy, 2002). This waste sludge is either disposed of in landfills or used for other purposes such as land application for farming due to its high nutrient content (Droste, 1997). Given that sorption to solids occurs readily with other hydrophobic compounds (Bondarenko & Gan, 2004; Thomas et al., 2009), it is likely that sorption can play a key role in the fate of VX and its hydrolysis products in an activated sludge system. If the compounds are not completely biodegraded within the plant, they will leave the wastewater treatment plants

in either the aqueous effluent, with the waste sludge, or both, thereby entering natural environmental systems.

Problem Statement

While several extensive studies have been conducted determining the mammalian toxicity and physical and chemical characteristics of the most prominent organophosphorous chemical warfare agents, current literature stops short of determining the fate of these compounds after on-site decontamination. The risks associated with an OP CWA attack are not limited to the scene of the incident as may be assumed. Given the stability and solubility of these agents, particularly VX, serious potential health concerns may exist beyond the point of treatment. Many communities plan for decontamination water to be treated in the local municipal wastewater treatment plant without knowing how these compounds will behave. If sorption occurs, toxic OPs may end up in waste sludge which is often land applied for fertilizer. If degradation does not occur, it is possible that OPs will leave the plant in the aqueous effluent. Both routes pose significant risk to local populace.

Research Questions

The purpose of this study was to determine experimentally the capacity of municipal wastewater treatment plant activated sludge (AS) to degrade EMPA, a hydrolysis product of the nerve agent, VX, in bench-scale studies. Additionally, this study aimed to determine the role of sorption to the activated sludge and its overall effect on the degradation of EMPA in bench-scale studies.

The primary goals of this study were to determine:

1. The degradation of EMPA by municipal wastewater treatment plant AS
 - a. The level of effectiveness AS has on biodegradation of varied EMPA concentrations
 - b. Degradation kinetics of AS with respect to EMPA
2. The role of sorption in waste sludge with respect to the fate of EMPA.

Scope and Approach

This research sought to simulate a municipal wastewater treatment plant aerobic digester in the laboratory by designing and operating a 2.0 L sequencing batch reactor. This sequencing batch reactor, seeded with activated sludge from the Fairborn Water Reclamation Facility (FWRF), Fairborn, Ohio and fed simulated wastewater, provided the activated sludge samples used in conducting batch test experiments.

Batch test experiments were completed to determine sorption characteristics of EMPA to activated sludge and the ability of activated sludge to degrade EMPA. The results provide insight into the fate of CWAs in a municipal wastewater treatment plant and the subsequent risk that may exist if compounds in question exit the plant unchanged.

Significance

In the event of a CWA incident, it is possible that decontamination wastewater could be sent to a wastewater treatment plant. If biodegradation is not complete, these compounds will leave a wastewater treatment plant in either the aqueous effluent, with the waste sludge, or both, thereby entering natural environmental systems. This pathway has the potential to pose significant human health concerns in the event OPs are introduced to a wastewater treatment facility, particularly in areas where treated

wastewater effluent eventually becomes a downstream potable water source. It is important to understand the behavior of these OP compounds in such biological systems in order to prevent the spread of OP contamination and human exposure to these toxic chemicals.

Preview

This thesis is written in the scholarly article format. Chapter 2 is a journal article produced from this research which is planned to be submitted to Water Environment Research. This article contains all necessary components prescribed by the peer review journal for submission. Written as an independent chapter it includes the following: abstract, introduction, materials and methods, results and discussion, and conclusions. Chapter 3 serves as a final discussion of the article conclusions. It also includes pertinent findings and identifies future research not discussed in Chapter 2.

II. Scholarly Article

Abstract

This study investigated the fate of ethyl methylphosphonic acid (EMPA), a hydrolysis product of VX, in a single sludge laboratory scale sequencing batch reactor (SBR). The reactor was fed peptone and sodium acetate to simulate wastewater. Sorption kinetics, sorption equilibrium isotherm and degradation batch experiments demonstrated that EMPA did not sorb to the biomass. Degradation results showed that approximately 28% of the initial concentration of 1 mg L⁻¹ EMPA was degraded. In addition, the results suggest that the nitrifying bacteria may be responsible for the degradation via cometabolism. Therefore, CWA may pass through an activated sludge wastewater treatment plant largely unchanged.

Keywords: Organophosphate chemical warfare agents, ethyl methylphosphonic acid, activated sludge

Introduction

Organophosphates (OPs) have been widely used as Chemical Warfare Agents (CWAs) as well as pesticides since World War II (Munro, Ambrose & Watson, 1994) and still remain a threat to national security. Although originally designed for military applications, these compounds have been used successfully against civilian populations in the past. While efforts have been taken at military installations and civilian communities to secure these chemicals and prevent their misuse, a determined adversary could still obtain and deploy them to injure, kill or instill terror. The lethal properties of this group of compounds are primarily owed to their irreversible inhibition of the enzyme

acetylcholinesterase (AChE) and thus may alter the human nervous system or affect the hormonal balance of children in particular (Pehkonen & Zhang, 2002). The most toxic organophosphorus CWAs include tabun (GA), sarin (GB), soman (GD), and VX.

In the event of a chemical incident, standard operating procedures dictate that contaminated personnel be decontaminated. Often times, decontamination is accomplished with water. Many communities plan for this decontamination water to be sent to the local municipal wastewater treatment plant. However, the fate of these compounds in a municipal wastewater treatment plant is largely unknown. If the compounds cannot be degraded, they will enter surface water bodies with plant effluent or waste sludge. Generally, degradation of OPs by hydrolysis is easily catalyzed, but the resulting alkyl methylphosphonate is likely to persist for years in the environment (Kingery & Allen, 1995).

Most municipal wastewater treatment plants in developed countries use activated sludge (bacteria) systems that might biodegrade the OPs. Furthermore, since these compounds are typically hydrophobic, they could likely sorb onto the biomass (Bondarenko and Gan, 2004). Therefore, the CWA compounds could either be transformed via biodegradation, removed with the waste activated sludge via sorption, or leave with the effluent if not degraded or sorbed.

VX was targeted in this research. Three of its physical properties set it apart as the worst case scenario for OP CWA exposure. First, with an LD₅₀ [dosage (milligrams toxicant per 70 kilogram person) causing death in 50% of an exposed population] of 10 mg/70Kg, VX is the most lethal of the nerve agents. Second, with a hydrolysis rate (half-life) of 1,000 hours, VX is the most persistent nerve agent. Lastly, with a volatility of

10.5 mg/m³, VX is the least likely to enter a vapor form. This is a key fact for this research as it is investigating the degradation of nerve agents in wastewater.

Because live agent testing was outside the scope and ability of our laboratory, EMPA, a hydrolysis product of VX, was used as a surrogate for VX during experimentation. In addition, decontamination procedures may include the use of bleach, which would raise the pH to above 10 and provide a VX degradation pathway favorable for the formation of EMPA. Therefore, EMPA in its own right could be encountered in real-world VX decontamination wastewater (Munro, 1999).

This work determined the capacity of municipal wastewater treatment plant activated sludge to degrade EMPA in bench scale studies. Additionally, it evaluated the role of sorption to the activated sludge and its overall effect on fate of EMPA in activated sludge.

Materials and Methods

Sequencing Batch Reactor Operation

The reactor was operated using a method adapted from Racz et al. (2010). A 2.0 L sequencing batch reactor (SBR) was constructed (Fig 4) and seeded with activated sludge from the Fairborn Water Reclamation Facility (FWRF), Fairborn, Ohio. Two feed sources (feed A and B) were used in order to maintain simultaneous chemical oxygen demand (COD) removal and nitrification. Feed A was a trace element solution and feed B consisted of a peptone/micronutrient mix, which simulated wastewater. Municipal wastewater is composed of a mixture of organic compounds, including volatile fatty

acids. In order to simulate the conditions of municipal wastewater, sodium acetate was added to represent the volatile fatty acids (Kindaichi et al., 2004) and peptone was added as complex organic carbon source (Goel & Noguera, 2006). Feed A contained (per liter) 44.6 g NaHCO_3 . Feed B contained the following (per liter): 6 g peptone, 1.25 g sodium acetate, 2.26 g NH_4Cl , 6.86 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.72 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.6675 g KH_2PO_4 and 20 mL of a trace element solution, adapted from Hesselmann et al. (1999). The trace element solution consisted of the following (per liter of deionized water): 5.46 g citric acid, 4.0 g hippuric acid, 0.72 g $\text{Na}_3\text{NTA} \cdot 2\text{H}_2\text{O}$, 0.3 g $\text{Na}_3\text{EDTA} \cdot 4\text{H}_2\text{O}$, 3.0 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.5 g H_3BO_3 , 0.3 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.24 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.14 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.06 g KI, 0.06 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.06 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.06 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.06 g $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$. Reactor operations consisted of two, 12 h cycles per day, consisting of two stages per cycle. Stage 1 began with a five minute filling sequence in which 624 mL deionized water, 38 mL feed A, and 8 mL feed B were added to the reactor via a peristaltic pump, bringing the total reactor volume to 2.0 L. The filling sequence was followed by an 11.5 h aerobic period in which the mixed liquor was aerated with compressed air to ensure adequate contact and maintain proper dissolved oxygen concentrations. Mixed liquor dissolved oxygen concentrations were maintained at approximately 7 mg L^{-1} . Aeration was turned off at the beginning of stage 2, followed by 20 minutes of settling, at which point, 670 mL was decanted (5 min). This 670 mL was then replaced at the beginning of the next cycle, yielding a 36 h hydraulic retention time. The solids retention time was 20 d.

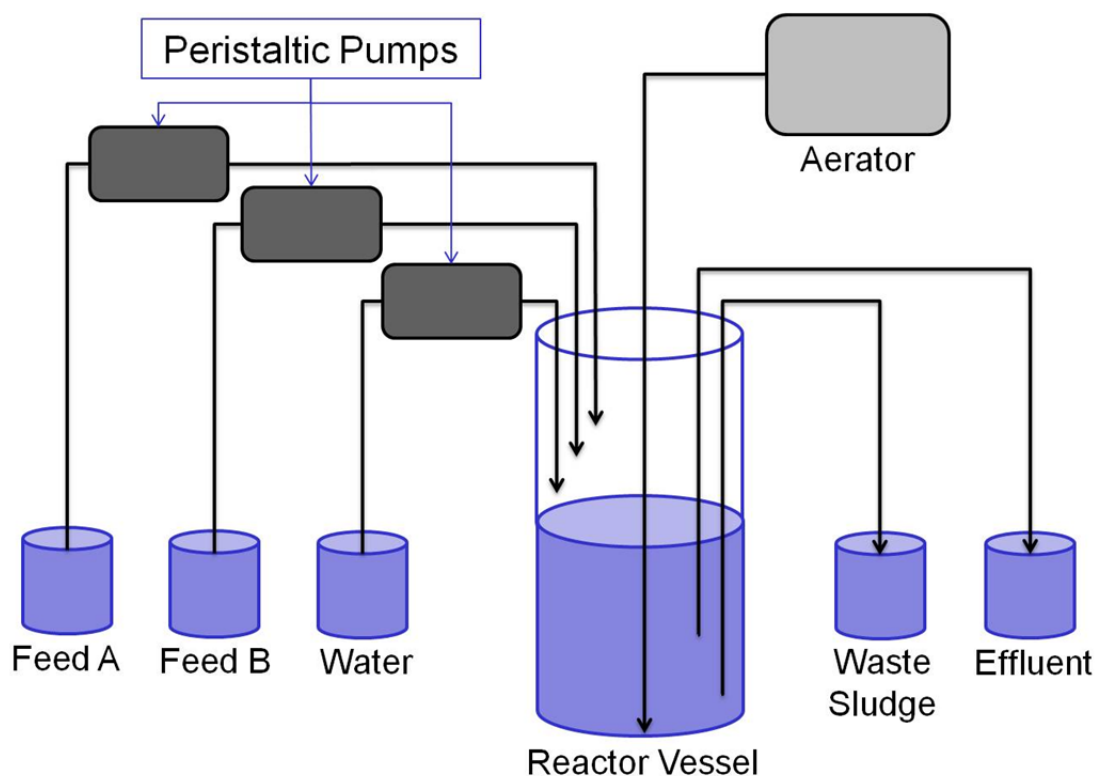


Figure 4: SBR setup

Solid and Liquid Phase

EMPA was extracted from both the solid and liquid phases of the biomass by passing a 10 mL sample through a Büchner funnel with a 1.2 μm Whatman GF/C glass fiber filter paper. The filtrate was collected in a syringe and further filtered using a 0.2 μm filter prior to analysis by UPLC/MS-MS. The GF/C filter paper containing the biomass solids was then placed in a beaker. Four mL methanol was added to the beaker, and beakers were covered with parafilm. Next, the beakers were sonicated for 10 minutes. After sonication, the liquid in the beaker was collected with a syringe, filtered with a 0.2 μm filter and analyzed by UPLC/MS-MS.

UPLC/MS-MS

Concentrations of EMPA in the samples were measured using a Waters Acquity ultra-performance liquid chromatography (UPLC) instrument with a 150 x 2.1 mm (3 μm particle size) Atlantis dC18 column and Waters tandem mass spectrometer (MS-MS). The UPLC/MS-MS was run in both ESI positive and negative modes with 2% formic acid and acetonitrile with an injection volume of 10 μL at a flow rate of 300 $\mu\text{L min}^{-1}$. Samples were held at the initial condition of 100% formic acid solution for 7 minutes. Acetonitrile was then added in a gradient from 45% to 60% from 7 to 8 minutes, followed by 100% formic acid solution from 8 to 10 minutes with a total run time of 10 minutes.

Sorption Kinetics

The purpose of the sorption kinetics experiment was to determine the amount of time necessary for maximum EMPA sorption onto the AS solids to occur. First, the AS was heat inactivated by placing it in the oven at 80° C for 30 minutes. At this temperature, the ribosomes of bacteria denature (Lee & Kaletunc, 2002) with minimal changes in sludge features (Ren et al., 2007b). While other studies have used sodium azide (NaN_3) to inactivate metabolic activity (Yi & Harper, 2007; Xu et al., 2008), NaN_3 selectively inhibits cytochrome oxidase in gram-negative bacteria. Gram-positive bacteria are resistant to the bacteriostatic effects of NaN_3 (Lichstein & Soule, 1943). Next, 8 mL of 1760 mg L^{-1} heat inactivated sludge and sufficient EMPA to bring the final concentration to 3 mg L^{-1} was added to each vial. The vials were then placed on a test

tube rotating disk. Vials were removed from the rotating disk at 5, 10, 20, 40, and 60 minutes at which point EMPA was extracted from the solid and liquid phases. Samples were compared to a control consisting of water and 3 mg L⁻¹ EMPA in order to account for the amount of EMPA sorbed to the filter paper. A two-tailed statistical analysis was conducted to determine if the percent of EMPA recovered from total suspended solids (TSS) was statistically different from the percent of EMPA recovered from filter paper. Total EMPA recovery for sorption kinetics and equilibrium as well as degradation experiments was calculated via the method outlined by Matuszewski et al. (2003). Sorption kinetics EMPA recoveries ranged from 83% to 93%. All measurements and tests were conducted in duplicate.

Sorption Isotherm

250 mL of heat-inactivated biomass (80°C for 30 min) was placed in Erlenmeyer flasks, each with a different concentration of TSS, namely 1235, 820, 795, 655, 585 and 175 mg L⁻¹. EMPA was added to each flask to a final concentration of 1 mg L⁻¹. The flasks were placed on stir plates for 20 min, a length of time at which sorption was considered complete according to the sorption kinetics results. The EMPA was extracted from the AS solid and liquid phases. Total EMPA recoveries ranged from 95% to 106%. All measurements and tests were conducted in duplicate.

Biodegradation

The purpose of the degradation experiment was to determine the capacity of activated sludge to degrade EMPA. This experiment was conducted with batch tests

using three separate flasks. Duplicate flasks contained AS, feed and EMPA. A control flask contained AS and feed, but no EMPA. Samples were taken each hour from the aerated AS flasks to measure concentrations of COD, ammonia, and EMPA from the AS solid and liquid phases. COD and ammonia were measured to monitor the performance of the AS heterotrophic and nitrifying bacteria. Measurements were conducted in duplicate.

Biodegradation with Inhibition of Nitrification

The purpose of this experiment was to determine the role of nitrifying bacteria in the degradation of EMPA by AS. This experiment was identical to the degradation experiment except that 86 μM (10 mg L^{-1}) allylthiourea (ATU) was added to the AS to inhibit nitrification. ATU was initially added 12 hours prior to the beginning of the experiment to ensure adequate time for nitrification inhibition. An additional 10 mg L^{-1} ATU was added just prior to the test start time in order to ensure inhibition of nitrifying bacteria for the duration of the experiment. ATU is believed to bind with the copper of the AMO active site (Bédard & Knowles, 1989), and therefore selectively inhibits nitrification. While ATU can inhibit nitrifiers at concentrations as low as 8 μM (Hoffman & Lees 1953; Hooper & Terry, 1973; Sharma & Ahlert, 1977; Tomlinson et al., 1966), complete inhibition can be achieved at an ATU concentration of 86 μM (10 mg L^{-1}) without affecting other metabolic activities (Ginestet et al., 1998). A fourth flask containing only water and EMPA served as another control to account for abiotic effects such as volatilization, losses to glassware, and losses during the extraction process. Measurements were conducted in duplicate.

Other Analytical Methods

Concentrations of COD, NH₃-N, NO₃⁻-N, and NO₂⁻-N were measured using Hach methods 8000, 10031, 10020, and 8153, respectively. TSS and volatile suspended solids (VSS) were measured using standard methods (APHA, AWWA, WEF, 1998). All measurements and tests were conducted in duplicate.

Results and Discussion

Sorption Kinetics and Equilibrium Isotherms

The intent of the sorption kinetics experiment was to determine the time required for maximum sorption of EMPA to inactivated sludge to occur. However, sorption to the biomass was statistically insignificant when compared to sorption on the filter paper during sample processing. The sorption equilibrium isotherm experiment evaluated whether sorption would change with varying TSS concentrations. These results similarly indicated that EMPA sorption for all TSS concentrations was no different than what was sorbed to the filter paper. Therefore, EMPA sorption is not an important removal mechanism in a municipal wastewater treatment plant. These results agree with the observation that the pK_a value of EMPA has been reported as 2.00 to 2.76 (Bossle et al. 1983), which predicts that EMPA will be highly dissociated in water, acidic and, therefore, less likely to sorb onto biomass.

Biodegradation

The liquid phase EMPA concentration decreased approximately 28% over the first 8 hours from a concentration of $975 \mu\text{g L}^{-1}$ to approximately $700 \mu\text{g L}^{-1}$ and remained relatively constant over the remaining 4 hours (Fig 5). The COD concentration decreased from 52 to 2 mg L^{-1} over 12 hours, which was similar to the control sample and indicated that there was active heterotrophic activity. Likewise, the $\text{NH}_3\text{-N}$ concentration decreased from 10.1 mg L^{-1} to 0.3 mg L^{-1} within 12 hours, indicating that nitrification was occurring. Therefore, the EMPA did not inhibit COD oxidation or nitrification activity.

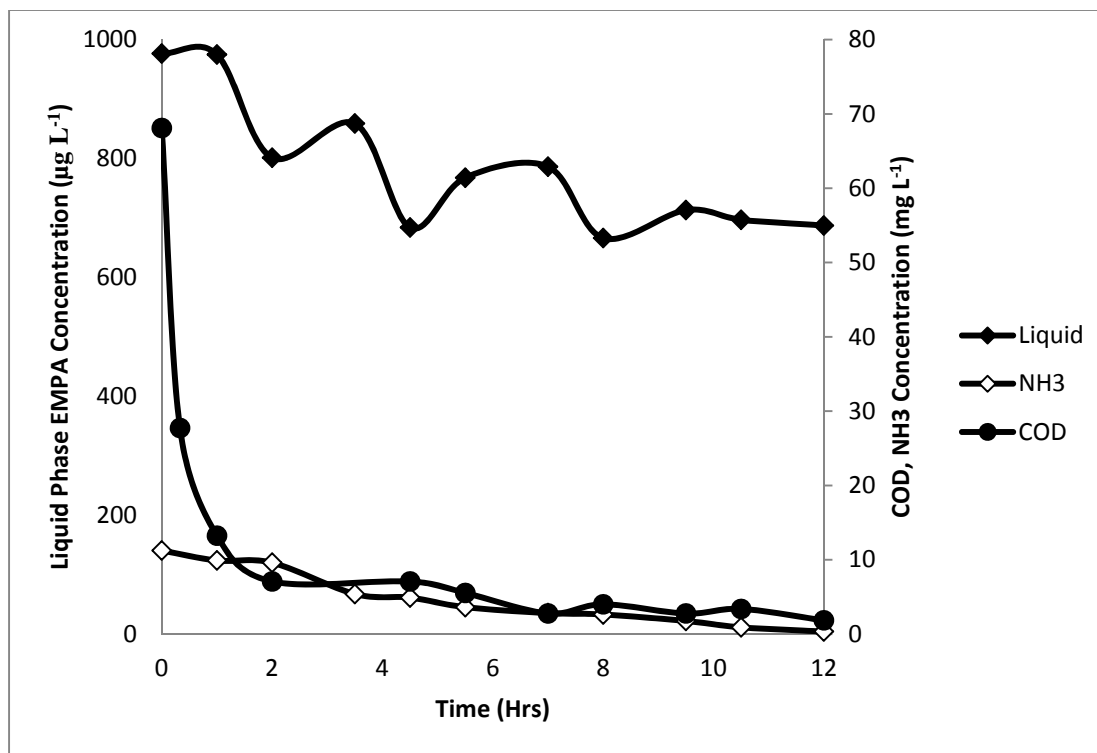


Figure 5: EMPA Biodegradation: 1 mg L^{-1} activated sludge at 25°C with nitrifiers active

To determine the role of the nitrifying bacteria, ATU was added to the flasks in the second degradation experiment (Fig 6). An additional flask containing water and EMPA was added to account for abiotic effects. The liquid phase concentration of EMPA remained unchanged throughout the duration of the 12-h experiment. Since the EMPA concentration in the liquid phase decreased in the presence of both heterotrophs and nitrifiers, but remained unchanged without nitrification activity, these observations suggest that nitrification activity may be responsible for EMPA degradation to some degree. Specifically, the ammonia monooxygenase enzyme involved with nitrification is known to degrade organic compounds via cometabolism (Ren et al., 2007a, Vader et al., 2000, Shi et al., 2004).

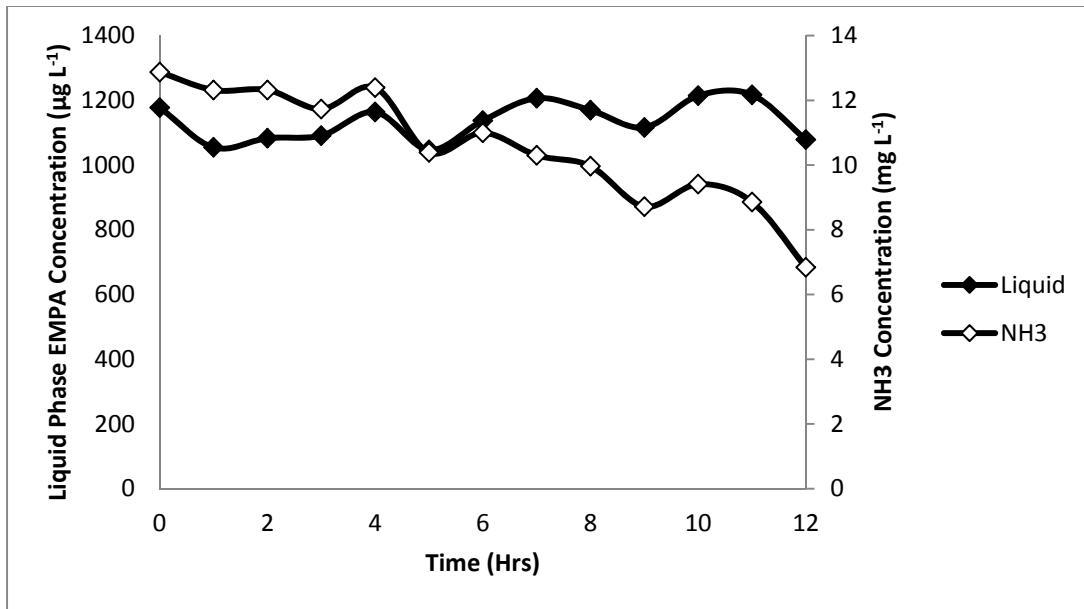


Figure 6: EMPA Biodegradation: 1.2 mg L⁻¹ activated sludge at 25° C with nitrifiers inhibited

While degradation of EMPA coincided with nitrification activity, no degradation occurred when NH₃ concentrations were below approximately 1 mg L⁻¹. There are two

potential causes for this lack of degradation. First, it is possible that there were insufficient concentrations of AMO available to continue to hydrolyze the EMPA. Second, it is possible that there is a threshold concentration below which EMPA can no longer be degraded in natural systems. A threshold effect, as previously described by Alexander (1985), is a concentration below which a substance cannot support bacterial growth. This observation may explain the persistence of low levels of biodegradable organic substances found in natural environments (Alexander, 1985). Therefore, it is unclear whether EMPA is inherently resistant or if it remains because of its low concentration.

Results show that if a CWA incident was to occur and if EMPA entered a municipal wastewater treatment plant, a large percentage of the EMPA would ultimately exit the WWTP in the effluent completely undegraded. If nitrification is being performed at the plant, some degradation may occur. No appreciable amount of EMPA would sorb to the activated sludge which mitigates the risk of EMPA leaving the plant with the waste biomass.

Conclusions

This study provides insight to the fate of EMPA in a municipal wastewater treatment plant and demonstrates that CWA may pass through an activated sludge wastewater treatment plant largely unchanged. Specifically, it was determined via sorption kinetics and isotherm experiments that any sorption of EMPA to AS is negligible. Additionally, we showed that only 28% of the initial 1 mg L⁻¹ EMPA was degraded and that nitrifying bacteria may be responsible for the degradation.

III. Conclusions

Chapter Overview

This chapter discusses the research findings which aimed to answer the research questions posed in Chapter 1. The results section serves as a summation to compliment the in depth discussion, included in the scholarly article, which is planned to be submitted to Water Environment Research. A brief discussion highlighting the significance of the research follows the review of findings. Finally, areas of future research are identified followed by an overall summary of the thesis.

Review of Findings

Our work demonstrates that CWA may pass through an activated sludge wastewater treatment plant largely unchanged. Specifically, the EMPA did not sorb to the biomass and degraded only about 28% at an initial concentration of 1 mg L^{-1} . In addition, the EMPA did not inhibit COD oxidation or nitrification activity in the activated sludge.

Significance of Research

In the event of a CWA incident, standard operating procedure dictates that decontamination be conducted with copious amounts of water. It is likely that this decontamination water will reach the local municipal wastewater treatment plant for treatment. Our research demonstrates that the majority of EMPA will pass through a wastewater treatment plant largely unchanged and exit the plant in the effluent.

Furthermore, if EMPA were to enter a municipal wastewater treatment plant that does not perform nitrification, there may be a higher concentration of EMPA in the effluent as nitrifying bacteria may responsible for some amount degradation, probably via cometabolism.

If the degradation and sorption characteristics of EMPA can be attributed to its parent compound, VX, or VX's highly lethal hydrolysis product, EA 2192, there would be significant concern for OP toxicity downstream of the wastewater treatment plant effluent, especially if the effluent eventually becomes a downstream potable water source.

Limitations

The first areas of limitations which cannot be ignored are those inherent to all lab based research. Lab conditions cannot possibly replicate the scale or complexity of field conditions. Therefore, results obtained cannot be directly applied to the field, but rather, generalized inferences are possible. This research focused on conducting a preliminary study to determine the fate of EMPA by activated sludge in a SBR. Therefore, this research is an approximation for field conditions for isotherm and degradation studies.

Another limitation is the fact that a surrogate, EMPA, was used. While it may be reasonable to assume that EMPA would be the hydrolysis product likely seen in a wastewater treatment plant, live agent testing would be more accurate and informative. Unfortunately, live agent testing of VX was simply beyond the scope of this project and AFIT's laboratories. As a result, it is important to realize that VX and EMPA will not

necessarily have the same chemical and physical behaviors and therefore, conclusions from EMPA cannot be directly applied to VX.

Future Research

The first area of future research is to determine how manipulation of the physical parameters of a municipal wastewater treatment plant will affect its ability to degrade EMPA. One such area for future research is determining the effect of increasing SRT on the degradation of EMPA. SRT directly relates to concentration of microorganisms and the amount of time the microorganisms are given to degrade compounds. In general, the longer the SRT, the lower the effluent concentration of a substrate compound (Rittmann & McCarty 2001). If adequate EMPA degradation is dependent on SRT, a critical value for the sludge age can be determined. Furthermore, if this dependence exists, degradation of EMPA would occur in WWTPs operating at SRTs higher than the critical value.

Second, while EMPA will likely be the hydrolysis product present given our research scenario, the same cannot be said for all conditions. If bleach is not used in decontamination, VX itself will likely be the most prevalent compound present. If hydrolysis occurs at a neutral pH, per the hydrolysis pathways discussed in the background, EA 2192 will be most prevalent. Due to these different possibilities, it is necessary to conduct similar sorption and degradation tests on both VX and EA 2192 to have a clearer understanding of the fate of VX in a municipal wastewater treatment plant.

Third, it is necessary to conduct degradation tests varying initial EMPA concentration to have a better determination on the theories of cometabolism and degradation threshold effects.

Summary

This research explored the fate of EMPA, a hydrolysis product of the OP CWA, VX, in a municipal wastewater treatment plant activated sludge system. The purpose of this research was to determine if EMPA would pass through an activated sludge system unchanged to identify possible significant human health concerns. The research methodology involved conducting laboratory batch tests using activated sludge grown in a sequencing batch reactor, seeded with sludge from the Fairborn Water Reclamation Facility. Data showed that sorption of EMPA to activated sludge does not occur and approximately 72% of the initial concentration of EMPA remained intact following degradation studies. Furthermore, it was determined that autotrophic, nitrifying bacteria may responsible for what degradation did occur, possibly via cometabolism. Future implications resulting from the research include a call for rethinking what should be done with decontamination wastewater in the event of a CWA incident. Overall, this research identifies the fact that the risks associated with a CWA attack are not limited to the incident site under current emergency planning procedures.

Appendix A. UPLC/MS-MS Calibration Curves

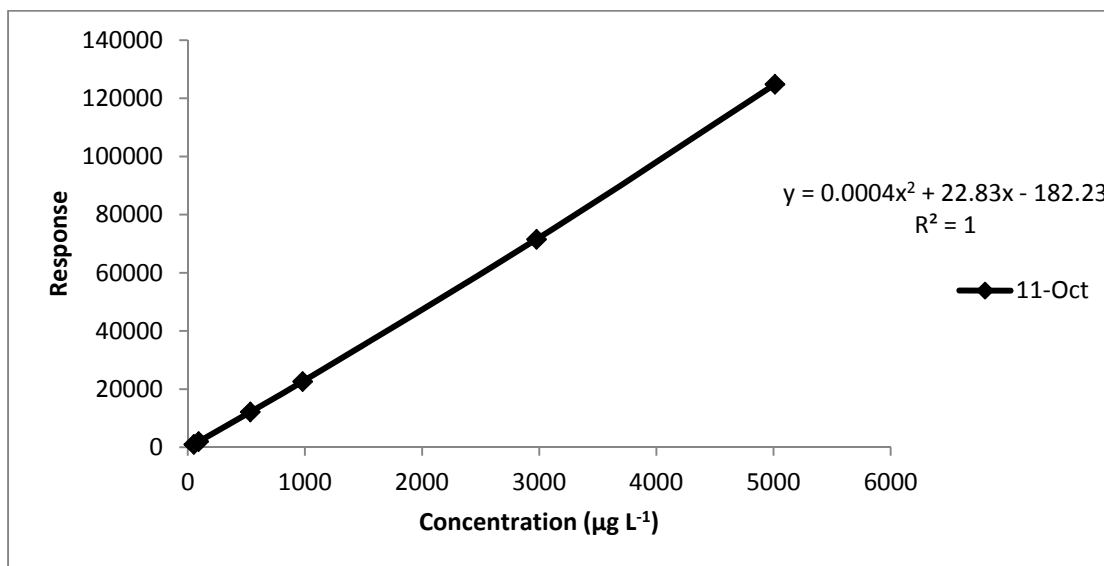


Figure 7: Calibration curve for sorption kinetics

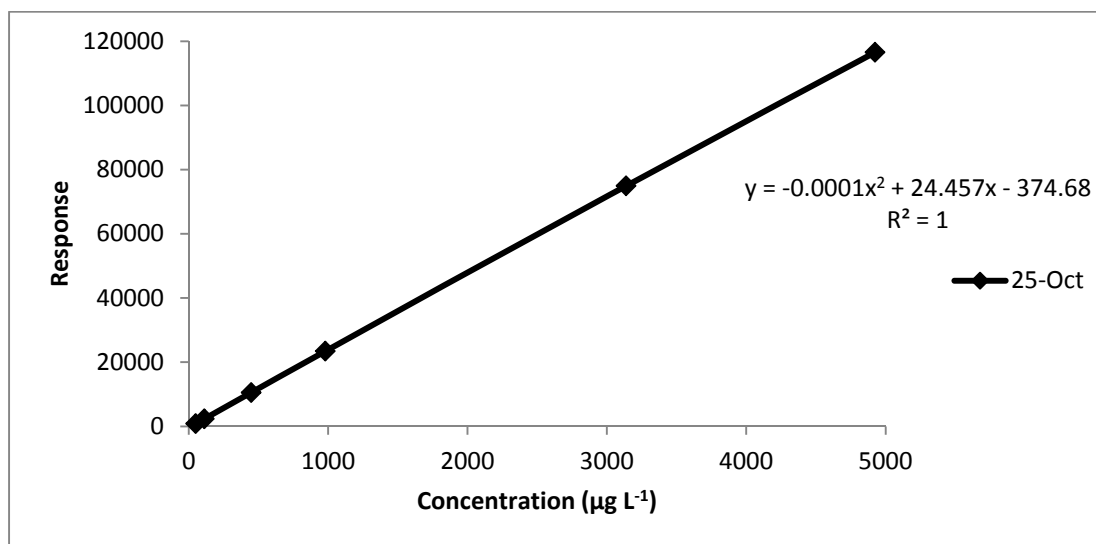


Figure 8: Calibration curve for sorption equilibrium isotherm

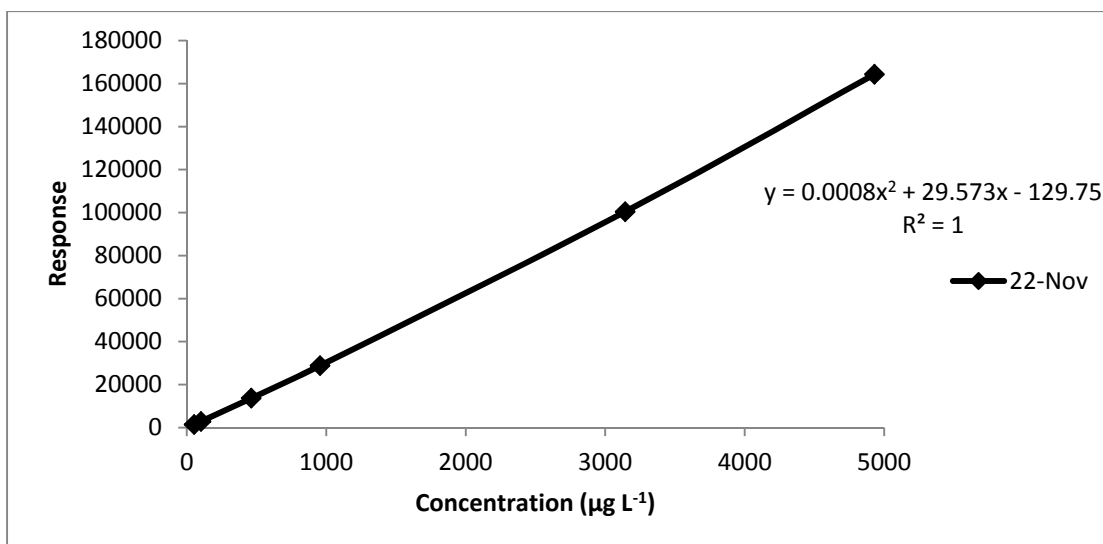


Figure 9: Calibration curve for degradation with nitrifiers active

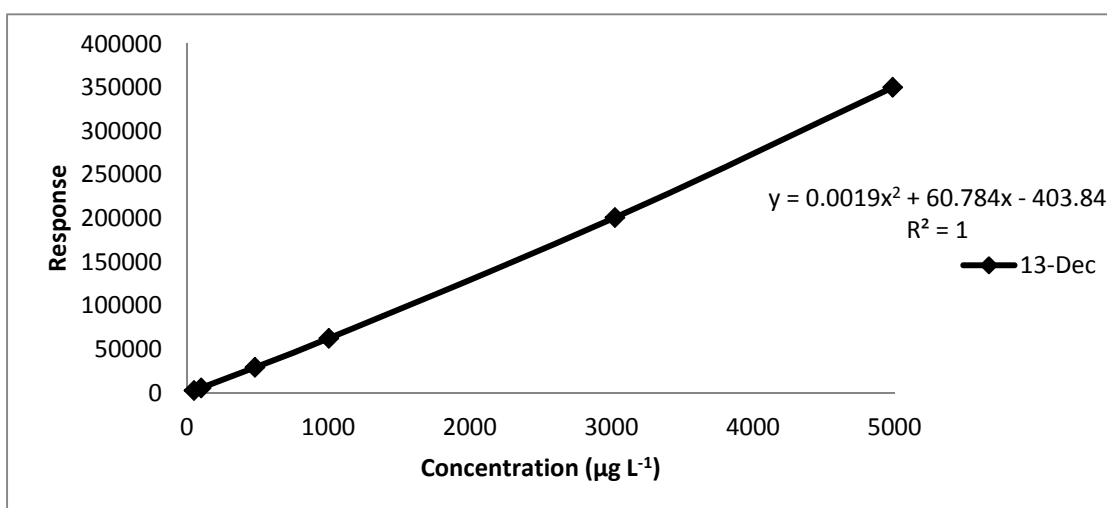


Figure 10: Calibration curve for degradation with nitrifiers inhibited

Bibliography

- Alexander, M. (1985). Biodegradation of organic chemicals. *Environmental Science & Technology*, 19(2), 106-111.
- APHA/AWWA/WEF 1998 *Standard Methods for the Examination of Water and Wastewater* (20th Edn), APHA/AWWA/WEF, Washington DC, USA.
- Associated press. Iraqi gas attack on Kurds disputed. Washington Times. December 18, 1990.
- Beaton R, Stergachis A, Oberle M, Bridges E, Nemuth M, Thomas T. The sarin gas attacks on the Tokyo subway-10 years later/ lessons learned. *Traumatology*. 2005;11(2):103-119.
- Bédard, C., & Knowles, R. (1989). Physiology, biochemistry, and specific inhibitors of CH₄, NH₄⁺, and CO oxidation by methanotrophs and nitrifiers. *Microbiological reviews*, 53(1), 68-84. Am Soc Microbiol.
- Bondarenko, S., & Gan, J. (2004). Degradation and sorption of selected organophosphate and carbamate insecticides in urban stream sediments. *Environmental Toxicology & Chemistry*, 23(8), 1809-1814.
- Bossle, P.C., Martin, J.J., Sarver, E.W., & Sommer, H.Z. (1983). High-performance liquid chromatography analysis of alkyl methylphosphonic acids by derivatization. *Journal of Chromatography*. 267:209-212
- Casarett, L, Doull, J., & Klaassen, C. (2008). Casarett and doull's toxicology: the basic science of poisons. New York, NY: McGraw-Hill Companies, Inc.
- Droste, R.L. (1997). Theory and practice of water and wastewater treatment. Hoboken, NJ: John Wiley & Sons, Inc.
- Dunn P. Chemical Aspects of the Gulf War, 1984-1987, Investigations by the United Nations. Ascot Vale, Australia: Materials Research Laboratories; 1987.
- Dunn P. The chemical war: journey to Iran. Nuclear, Biological, and Chemical Defense and Technology International. 1986; 28-35
- Fox, S. (2009). Human physiology. New York, NY: McGraw-Hill
- Gallo M.A., Lawryk N.J.: Organic phosphorus pesticides, in Hayes WJ, Laws ER (Eds.): Handbook of Pesticide Toxicology. San Diego: Academic Press, 1991, pp. 917-1123.

- Ginestet, P., Audic, J.-M., Urbain, V., & Block, J.-C. (1998). Estimation of nitrifying bacterial activities by measuring oxygen uptake in the presence of the metabolic inhibitors allylthiourea and azide. *Applied Environmental Microbiology*, 2266-2268.
- Goel, R.K. & Noguera, D.R. (2006). Evaluation of sludge yield and phosphorous removal in a Cannibal solids reduction process. *Journal of Environmental Engineering*, 132(10), 1331-1337.
- Hesselmann, R.P.X., Werlen, C., Hahn, D., van der Meer, J.R. & Zehnder, A.J.B. (1999). Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge. *Systems Applied Microbiology* 22, 454-465
- Hill, B. A. Jr. Hilmas, C.J. & Smart, J.K (2008). History of Chemical Warfare. In J. Redding, R., V. Mason, M. Metzgar, D. Wise, B. Maston (Eds.), Textbooks of Military Medicine: Medical Aspects of Chemical warfare (pp.62,63) Fort Sam Houston, Texas: US Army Medical Department Center and School & Office of The surgeon General Department of the Army, United States of America.
- Hill, B. A. Jr. Kok, A.B. Mauroni, Al. & Smart J.K. (2008). History of the Chemical Threat, Chemical Terrorism, and Its implications for military Medicine. In J. Redding, R. Lindsay, V. Mason, M. Metzgar, D. Wise, B. Maston (Eds.), Textbooks of Military Medicine: Medical Aspects of Chemical warfare (pp.126-128) Fort Sam Houston, Texas: US Army Medical Department Center and School & Office of The surgeon General Department of the Army, United States of America.
- Hoffman MS, ed. *The World Almanac and Book of Facts*, 1990. New York, NY: Pharaoh Books; 1990: 44, 46, 49, 721
- Hoffman, T., & Lees, H. (1953). The biochemistry of nitrifying organisms. 4. The respiration and intermediary metabolism of Nitrosomonas. *Biochemistry Journal*, 579-583.
- Holmstedt, B. (1963). Structure-activity relationships of the organophosphorus anticholinesterase agents. In: Koelle, G.B. (ed.), Handbuch Experimenteller Pharmakologie, Ergänzungswerk XV, Cholinesterases and Anticholinesterase Agents. Springer Verlag, Berlin.
- Hooper, A. B., & Terry, K. R. (1973). Specific inhibitors of ammonia oxidation in Nitrosomonas. *Bacteriol*, 480-485.
- Hyman, M. R., Murton, I. B., & Arp, D. J. (1988). Interaction of Ammonia Monooxygenase from Nitrosomonas europaea with Alkanes, Alkenes, and Alkynes. *Applied and Environmental Microbiology*, 54(12), 3187-3190.

- Hyman, M. R., & Wood, P. M. (1983). Methane oxidation by *Nitrosomonas europaea*. *The Biochemical journal*, 212(1), 31-37
- Katzung BG, Masters SB, Trevor AJ. Basic and clinical pharmacology. 12th ed. New York, NY: McGraw-Hill Medical; 2011.
- Kindaichi, T., Ito, T., & Okabe, S. (2004). Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. *Applied and Environmental Microbiology*, 70(3), 1641-1650. American Society for Microbiology
- Kingery AF, Allen HE, (1995). The environmental fate of organophosphorus nerve agents: a review, *Toxicological and environmental chemistry* 47:155-184.
- Lee, J., & Kaletunc, G. (2002). Evaluation of the Heat Inactivation of *E. coli* and *Lactobacillus planatarum* by Differential Scanning Calorimetry. *Applied Environmental Microbiology*, 5379-5386.
- Lichsterin, H. C., & Soule, M. H. (1943). Studies of the effect of sodium azide on microbic growth and respiration. *Bacteriol*, 221-230.
- Lotti, M: Organophosphorus compounds, in Spencer PS, Schaumburg HH, Ludolph AC (Eds): *Experimental and Clinical Neurotoxicology*. Oxford University Press, 2000, pp. 898-925.
- Lotti M: Clinical Toxicology of anticholinesterases in humans, in Krieger R (Ed): *Handbook of Pesticide Toxicology*. San Diego: Academic Press, 2001, pp. 1043-1085.
- Matuszewski, B.K., Constanzer, M.L., & Chavez-Eng, C.M. (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*, 3019-3030.
- McDonough, J.H., Newmark, J., & Sidell, F.R. (2008) Nerve Agents. In J. Redding, R. Lindsay, V. Mason, M. Metzgar, D. Wise, B. Maston (Eds.), *Textbooks of Military Medicine: Medical Aspects of Chemical Warfare* (pp.197) Fort Sam Houston, Texas: US Army Medical Department Center and School & Office of The surgeon General Department of the Army, United States of America.
- Metcalf, & Eddy (2002). *Wastewater engineering treatment and reuse*. McGraw Hill Higher Education.
- Munro, N.B., Ambrose, K.R., & Watson, A.P. (1994). Toxicity of the organophosphate chemical warfare agents GA, GB, and VX: implication for public protection. *Environ Health Perspect* 102:18-38.

- Munro, N. B., Talmage, S. S., Griffin, G. D., Waters, L. C., Watson, A. P., King, J. F., & Hauschild, V. (1999). The sources, fate, and toxicity of chemical warfare agent degradation products. *Environ Health Perspect*, 107(12)
- Ohio State University Extension. "Wastewater Treatment Principles and Regulations." Excerpt from unpublished article. n. pag. <http://ohioline.osu.edu/aex-fact/0768.html>.
- Pehkonen SO, Zhang Q, 2002, The degradation of organophosphorus pesticides in natural waters: a critical review, *Cri. Rev. Env. Sci. Tech* 32:17-32.
- Purver R. Chemical and Biological Terrorism: The Threat According to the Open Literature. Canadian Intelligence Security Service unclassified report. June 1995.
- Recollecting the series of Aum Shinrikyo incidents. August 30, 1996. Monterey Institute of International Studies Web site. Available at <http://cns.mii.edu/pubs/eanp/wapa.pdf>. Accessed May 15, 2008
- Racz, L., T. Datta, and Goel, R.K., (2010). Organic Carbon Effect on Nitrifying Bacteria in a Mixed Culture, *Water Science and Technology*, 61(11):2951-2956
- Ren, Y.-X., Nakano, K., Nomura, M., Chiba, N., & Nishimura, O. (2007). Effects of bacterial activity on estrogen removal in nitrifying activated sludge. *Water Research*, 41(11), 3089-3096.
- Ren, Y.-X., Nakano, K., Nomura, M., Chiba, N., & Nishimura, O. (2007). A thermodynamic analysis on adsorption of estrogens in activated sludge process. *Water Research*, 41(11), 2341-2348.
- Richardson, D. D., & Caruso, J. A. (2007). Derivatization of organophosphorus nerve agent degradation products for gas chromatography with ICPMS and TOF-MS detection. *Analytical & Bioanalytical Chemistry*, 388(4), 809-823.
- Rittmann, B.E., McCarty, P.L., 2001. *Environmental Biotechnology: Principles and Applications*. McGraw-Hill Higher Education, New York.
- Robinson, J.P., & Leitenberg, M. (1971). The rise in CB weapons. In: *The Problem of Chemical and Biological Warfare*. Almquist & Wiksells, Stockholm.
- Rohrbaugh DK, Ward JR, Yang Y. Comments on the Origin of Mustard in the Gulf War. Aberdeen Proving Ground, Md: US Army Chemical Research Development and Engineering Center; 1990: 8. Technical Report.
- Sharma, B., & Ahlert, R. C. (1977). Nitrification and nitrogen removal. *Water Resources*, 897-925.

- Smithson, A, Levy L. Rethinking the lessons of Tokyo. In: Smithson A, Levy Eds. Ataxia, the Chemical and Biological Terrorism threat and the U.S response. Stimson Center Report No. 35; 2000. Henry L Stimson Center Web Site. Available at <http://www.stimson.org/cbw/pdf/fullpagehtml>. Accessed May 15, 2008
- Spiers EM. Chemical Weaponry: A Continuing Challenge. New York, NY: St. Martin's Press; 1989: 212.
- Shi, J., Fujisawa, S., Nakai, S., & Hosomi, M. (2004). Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Water Research*, 38(9), 2322-2329.
- Szinicz, L. (2005). History of chemical and biological warfare agents. *Toxicology*, 214(3), 167-181. doi:DOI: 10.1016/j.tox.2005.06.011
- Talmage, S. S., Watson, A. P., Hauschild, V., Munro, N. B., & King, J. (2007). Chemical warfare agent degradation and decontamination. *Current Organic Chemistry*, 11(3), 285-298.
- Thomas, S. M., Bodour, A. A., Murray, K. E., & Inniss, E. C. (2009). Sorption behavior of a synthetic antioxidant, polycyclic musk, and an organophosphate insecticide in wastewater sludge. *Water Science and Technology*, 60(1), 145-154.
- Tomlinson, T. G., Boon, A. G., & Trotman, C. N. (1966). Inhibition of nitrification in the activated sludge process of sewage disposal. *Applied Bacteriol* , 266-291.
- United Nations. Report of the Mission Dispatched by the Secretary-General to investigate Allegations of the Use of Chemical Weapons in the conflict Between the Islamic Republic of Iran and Iraq. New York, NY: United Nations Security Council. March 12, 1986:19.
- U.N panel says Iraq used gas on civilians. New York Times. August 24, 1988.
- Vader, J. S., Van Ginkel, C. G., Sperling, F. M., De Jong, J., De Boer, W., De Graaf, J. S., Van Der Most, M., et al. (2000). Degradation of ethinyl estradiol by nitrifying activated sludge. *Chemosphere*, 41(8), 1239-1243
- Xu, K., Harper, W. F. Jr., & Zhao, D. (2008). 17 α -Ethinylestradiol sorption to activated sludge biomass: Thermodynamic properties and reaction mechanisms. *Water Res.* 42, 3146, 3152.
- Yi, T., & Harper, W. F. (2007). The link between nitrification and biotransformation of 17 a-ethinylestradiol. *Environmental Science Technology* , 4311-4316.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 074-0188	
<p>The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of the collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.</p> <p>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</p>					
1. REPORT DATE (DD-MM-YYYY) 22-03-2012		2. REPORT TYPE Master's Thesis		3. DATES COVERED (From – To) November 2010 – March 2012	
4. TITLE AND SUBTITLE Biodegradation of Organophosphate Chemical Warfare Agents by Activated Sludge				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Schuldt, Steven J., Captain, USAF				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(S) Air Force Institute of Technology Graduate School of Engineering and Management (AFIT/ENV) 2950 Hobson Way, Building 640 WPAFB OH 45433-8865				8. PERFORMING ORGANIZATION REPORT NUMBER AFIT/GES/ENV/12-M04	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Intentionally left blank				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED					
13. SUPPLEMENTARY NOTES This material is considered a work of the U.S. Government and is not subject to copyright protection in the United States					
14. ABSTRACT This study investigated the fate of ethyl methylphosphonic acid (EMPA), a hydrolysis product of VX, in a single sludge laboratory scale sequencing batch reactor (SBR). The reactor was fed peptone and sodium acetate to simulate wastewater. Sorption kinetics, sorption equilibrium isotherm and degradation batch experiments demonstrated that EMPA did not sorb to the biomass. Degradation results showed that approximately 28% of the initial concentration of 1 mg L ⁻¹ EMPA was degraded. In addition, the results suggest that the nitrifying bacteria may be responsible for the degradation via cometabolism. Therefore, CWA may pass through an activated sludge wastewater treatment plant completely unchanged.					
15. SUBJECT TERMS Organophosphate chemical warfare agents, ethyl methylphosphonic acid, activated sludge					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Racz, LeeAnn, Maj, Ph.D., USAF
U	U	U	UU	50	19b. TELEPHONE NUMBER (Include area code) (937) 255-6565, x 4711 (leeann.racz@afit.edu)